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Pet-1 controls tetrahydrobiopterin pathway and *Slc22a3* transporter genes in serotonin neurons

Steven C. Wyler[*], **Lauren J. Donovan**[*], **Mia Yeager**, and **Evan Deneris**[#]

Department of Neurosciences, Case Western Reserve University Cleveland, Ohio, 44106, United States

Abstract

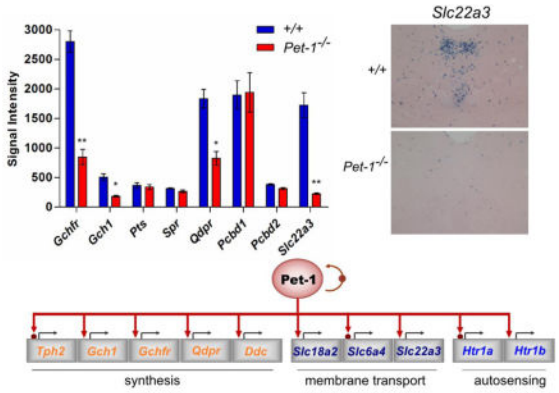
Coordinated serotonin (5-HT) synthesis and reuptake depends on coexpression of Tph2, Aadc *(Ddc)* and Sert *(Slc6a4)* in brain 5-HT neurons. However, other gene products play critical roles in brain 5-HT synthesis and transport. For example, 5-HT synthesis depends on coexpression of genes encoding the enzymatic machinery necessary for the production and regeneration of tetrahydrobiopterin (BH4). In addition, the organic cation transporter 3 (Oct3, *Slc22a3*) functions as a low affinity, high capacity 5-HT reuptake protein in 5-HT neurons. The regulatory strategies controlling BH4 and Oct3 gene expression in 5-HT neurons have not been investigated. Our previous studies showed that Pet-1 is a critical transcription factor in a regulatory program that controls coexpression of Tph2, Aadc and Sert in 5-HT neurons. Here, we investigate whether a common regulatory program determines global 5-HT synthesis and reuptake through coordinate transcriptional control. We show with comparative microarray profiling of flow sorted YFP^{+} *Pet-1*$^{-/-}$ and wild type 5-HT neurons that Pet-1 regulates BH4 pathway genes, *Gch1*, *Gchfr*, and *Qdpr*. Thus, Pet-1 coordinates expression of all rate-limiting enzymatic (*Tph2*, *Gch1*) and post-translational regulatory (*Gchfr)* steps that determine the level of mammalian brain 5-HT synthesis. Moreover, Pet-1 globally controls acquisition of 5-HT reuptake in dorsal raphe 5-HT neurons by coordinating expression of *Slc6a4* and *Slc22a3. In situ* hybridizations revealed that virtually all 5-HT neurons in the dorsal raphe depend on Pet-1 for *Slc22a3* expression; similar results were obtained for *Htr1a*. Therefore, few if any 5-HT neurons in the dorsal raphe are resistant to loss of Pet-1 for their full neuron-type identity.

Abstract

[#]Corresponding author: 216-368-8725, esd@case.edu.
[*]equal contribution.

Author contributions

SCW, LJD, ED conceived and designed the experiments. SCW, LJD, MY performed the experiments. SCW, LJD, ED analyzed the data and wrote the manuscript.



Keywords

Serotonin; tetrahydrobiopterin; Organic cation transporter 3; Pet-1; GTP cyclohydrolase I; GTP cyclohydrolase I feedback regulator; Quinoid dihydropteridine reductase

Coexpression of unique gene combinations encoding numerous kinds of neuron-type and pan neuronal characteristics establishes the identity of different neurons [(1)]. However, the gene regulatory mechanisms controlling the acquisition of neuron-type identities are poorly understood. One key and obvious identity feature that distinguishes different neuron types is transmitter identity. Transmitter identity is commonly defined by the presence of a particular transmitter together with the coexpression of genes required for its synthesis, reuptake, and vesicular transport in specific neuron-types [(2)]. In the case of 5-HT neurons, the gene products that typically define serotonergic transmitter identity are tryptophan hydroxylase 2 (Tph2), aromatic amino acid decarboxylase (Aadc, gene symbol *Ddc*), serotonin transporter (Sert, gene symbol *Slc6a4*), vesicular monoaminergic transporter 2 (Vmat2, gene symbol *Slc18a2*) and the 5-HT1a (gene symbol *Htr1a*) and 5-HT1b (gene symbol *Htr1b*) autoreceptors.

A serotonergic gene regulatory network, comprising multiple interacting transcription factors, has been identified that coordinates expression of Tph2, Aadc, Sert, Vmat2, 5-HT1a, and 5-HT1b in brain 5-HT neurons [(3)]. Transcription factors Ascl1, Nkx2.2, and Foxa2 are required for specification of serotonergic progenitors in the ventral hindbrain. These factors subsequently activate a downstream transcription factor network comprising, Gata-2, Insm1, Gata3, Lmx1b, Engrailed1/2, and Pet-1, which acts in postmitotic serotonergic precursors to initiate 5-HT neuron-type differentiation [(4–9)]. Germ line targeting of each of these factors results in aborted differentiation to varying extents depending on which factor is missing [(3)]. For example, the Pet-1 ETS factor is required for coordinate expression of *Tph2*, *Ddc*, *Slc6a4*, *Slc18a2*, *Htr1a*, and *Htr1b* in postmitotic serotonergic precursors as expression of each of these 5-HT identity genes is severely reduced in *Pet-1*$^{-/-}$ 5-HT neurons [(7, 10, 11)]. *In vivo* chromatin immunoprecipitation and *in vitro* DNA binding assays have demonstrated that Pet-1 coordinates expression of these serotonergic genes through direct binding to a common conserved ETS DNA binding site in their promoter regions [(11, 12)]. Although Pet-1 is expressed in what appears to be all brain 5-HT neurons, Tph2 continues to be expressed,

albeit at reduced levels, in a subset of *Pet-1*$^{-/-}$ 5-HT neurons suggesting the presence of a Pet-1 resistant subpopulation of 5-HT neurons [7, 10, 13].

In addition to the genes described above, other gene products play critical roles in 5-HT synthesis and transport and therefore are necessary for 5-HT to function as a transmitter. For example, in addition to Tph2 and Aadc, 5-HT synthesis depends on coordinate expression of the enzymatic machinery catalyzing the production and regeneration of 6R-L-erythro-5,6,7,8-tetrahydrobiopterin (BH4), an obligatory cofactor for Tph2 enzymatic activity as well as the enzymatic activity of other monoaminergic monooxygenases, nitric oxide synthases, and alkylglycerol monooxygenase [14–17]. BH4 is synthesized *de novo* from the precursor guanosine triphosphate (GTP) in four or five enzymatic steps (Figure 1) catalyzed by GTP cyclohydrolase I (Gtpch, gene symbol *Gch1*), 6-pyruvoyltetrahydropterin synthase (Ptps, gene symbol *Pts*), and Sepiapterin reductase (SR, gene symbol *Spr*). The enzymatic steps catalyzed by SR, however, can be alternatively catalyzed by aldo-keto-reductase family 1 member 3 (mouse ortholog *Akr1c18*), aldo-keto-reductase family 1 B1 (mouse ortholog *Akr1b3*), and carbonyl reductase (CR, gene symbol *Cbr1*) in various combinations (Figure 1) [14]. Although, it is commonly accepted that Tph2 is a rate-limiting step for the production of 5-HT, Gtpch activity is rate limiting for BH4 synthesis and therefore control of its expression level is a critical determinant of 5-HT synthesis. Gtpch enzymatic activity is also controlled post-translationally through negative feedback regulation by GTP cyclohydrolase I feedback regulatory protein (Gfrp, gene symbol *Gchfr*) [18, 19]. The allosteric binding of BH4 to Gtpch stimulates the formation of a multimeric Gfrp:Gtpch complex in which Gtpch activity is inhibited [19, 20]. In contrast, L-phenylalanine can stimulate BH4 biosynthesis in a Gfrp-dependent manner [21]. After its enzymatic conversion to 4α-hydroxy-tetrahydrobiopterin in a monooxygenase or synthase reaction, BH4 can be regenerated in a two-step pathway catalyzed by pterin-4α-carbinolamine dehydratase (PCD, gene symbol *Pcbd1* and *Pcbd2*) and dihydropteridine reductase (Dhpr, gene symbol *Qdpr*, quinoid dihydropteridine reductase) [15]. A large number of rare mutations, causing BH4 and monoamine deficiency, have been identified in human *GCH1*, *PTS*, *SPR*, *PCBD1*, and *QDPR* genes and are responsible for several neurological motor control disorders such as dopa-responsive dystonia or Segawa disease [22]. The 5-HT transporter, Sert (gene symbol *Slc6a4*), is responsible for high affinity reuptake of 5-HT [23, 24]. However, other transporters are now recognized as playing important roles in clearing 5-HT from the synaptic cleft and extrasynaptic sites [25]. For example, Oct3 (gene symbol *Slc22a3*) has been shown to function as a low affinity, high capacity 5-HT transporter [26]. Oct3 expression is abundant in brain 5-HT neurons and importantly it is a critical determinant of SSRI efficacy [27]. However, the regulatory mechanisms that control expression of BH4 and *Slc22a3* genes in 5-HT neurons have not been investigated.

A possibility is that a regulatory network distinct from that controlling *Tph2*, *Ddc*, *Slc6a4*, *Slc18a2*, *Htr1a*, and *Htr1b*, controls these additional key serotonergic genes. This possibility seems plausible as BH4 enzymatic pathways are expressed in numerous neural and non-neural cell types of the brain and periphery and are required for other cellular functions in addition to 5-HT synthesis [14, 15]. Similarly, Oct3 is widely expressed in many different

neuron-types and glia of the adult rodent brain unlike Tph2, Sert, and Vmat2 [28, 29]. Alternatively, as for other neural expressed genes [30, 31] the complex expression patterns of Oct3 and BH4 genes might be generated through separate cis regulatory control modules one of which positively responds to the same transcription factors that control other serotonergic genes such as Tph2.

To begin to understand the regulatory mechanisms that control Oct3 expression and BH4 production in mouse 5-HT neurons, we investigated whether their serotonergic expression is controlled by Pet-1. We previously reported a microarray method for transcriptome studies of flow sorted yellow fluorescent protein (YFP) -expressing fetal 5-HT neurons obtained from the ePet-EYFP transgenic mouse line [32, 33]. Here, we present a protocol for flow sorting of ePet-EYFP-marked *Pet-1*$^{-/-}$ 5-HT neurons from the fetal rostral hindbrain. We used this new protocol for comparative microarray analyses of *Slc22a3* and BH4 gene expression in wild type and *Pet-1*$^{-/-}$ 5-HT neurons. In addition, we investigated the relative Pet-1 dependency of serotonergic genes in the adult dorsal raphe.

Results and Discussion

In previous histochemical studies, we found comparable numbers of *Pet-1*$^{-/-}$ 5-HT neuron cells bodies and wild type 5-HT neuron cell bodies in the midbrain dorsal raphe [34]. Here, we crossed *ePet-EYFP*$^{+/+}$ and *ePet-EYFP*$^{Pet\text{-}1-/-}$ mice to generate *ePet-EYFP*$^{Pet+/-}$ offspring. These offspring were then interbred to generate *ePet-EYFP*$^{+/+}$ and *ePet-EYFP*$^{Pet\text{-}1-/-}$ littermate embryos. Anti-YFP immunostaining of fetal 5-HT neurons in the *ePet-EYFP*$^{+/+}$ and *ePet-EYFP*$^{Pet\text{-}1-/-}$ brain revealed comparable levels of YFP expression and similar numbers of wild type and mutant neurons (Figure 2A, B). These findings suggested we should be able to sort sufficient numbers of *Pet-1*$^{-/-}$ 5-HT neurons for microarray gene expression profiling. Embryonic (E) 12.5 YFP^{+} rostral hindbrain domains were dissected, dissociated and purified by flow cytometry as previously described [33]. Because Pet-1 may regulate the rostral and caudal 5-HT system differently, we only used tissue form the rostral 5-HT system which gives rise to the dorsal and median raphe and B9 nuclei.

Both +/+ and *Pet-1*$^{-/-}$ 5-HT neurons were readily sorted (Figure 2C–F) and comparable numbers were obtained (Figure 2G). RT-qPCR revealed, as expected, a complete lack of *Pet-1* expression in YFP^{+} RNA isolated from *Pet-1*$^{-/-}$ rostral hindbrain (Figure 2H). Moreover, *Tph2* and *Slc6a4* RNA levels were also dramatically reduced in *Pet-1*$^{-/-}$ embryos compared to control levels as expected [7]. Importantly, the expression of *Lmx1b* a serotonergic transcription factor whose expression is independent of Pet-1 at fetal stages was unchanged [5]. Thus, flow sorted Pet-1$^{-/-}$ YFP^{+} 5-HT neurons can be used to determine the impact of Pet-1 loss of function on *Slc22a3* and BH4 gene expression.

Having established a protocol for flow cytometry of *Pet-1* mutant 5-HT neurons we set up crosses to generate sufficient numbers of embryos to perform four microarray biological replicates for both *Pet-1*$^{-/-}$ and +/+ 5-HT neurons. Based on our previous studies [33] we collected between 20 and 30 thousand cells per replicate. This yielded sufficient RNA to

generate labeled cDNA probes, which were then hybridized to the GeneChiP Mouse Gene 1.0 ST array.

As a validation of our microarray approach, we first examined *Pet-1* probe intensities in arrays hybridized with +/+ and *Pet-1*$^{-/-}$ cDNA (Figure 3A). Analysis of probe intensities revealed background levels of *Pet-1* expression. As a further test of our approach we examined expression levels of several known *Pet-1* downstream targets. Expression levels of *Tph2*, *Ddc*, *Slc6a4*, *Slc18a2*, and *Htr1a* were all significantly reduced in *Pet-1*$^{-/-}$ vs. +/+ arrays (Figure 3A) to extents that correlate well with previous histochemical studies of these genes in *Pet-1*$^{-/-}$ mice. We also note that expression of *Lmx1b*, a gene not regulated by Pet-1 in fetal 5-HT neurons [5, 11], was not altered in the present microarray analysis of its expression in mutant 5-HT neurons. Our array findings indicated very low expression of *Hrt1a* and *Hrtr1b*, which is consistent with our earlier findings that expression of these two autoreceptor genes is not strongly induced until after E14 [11]. Thus, the very low, near background levels, of these genes at E12.5 likely precluded detection of *Htr1b*'s Pet-1 dependency although *Htr1a* did show significantly reduced expression in sorted *Pet-1*$^{-/-}$ 5-HT neurons (Figure 3B). Importantly, our array findings for *Tph2*, *Ddc*, *Slc6a4*, *Slc18a2*, *Htr1a*, and *Lmx1b* were perfectly consistent with our previously published studies of their Pet-1 dependency [35].

Having demonstrated the validity of our array approach for reproducible and accurate detection of gene expression changes in response to loss of Pet-1 we analyzed our datasets for expression of *Slc22a3* and BH4 genes (Figure 3B). Examination of the +/+ arrays revealed that in comparison to *Pet-1* and other 5-HT genes, *Slc22a3*, *Gchfr*, *Qdpr*, and *Pcbd1* are robustly expressed at E12.5. *Gchfr* was the most abundantly expressed BH4 gene in our dataset, which is consistent with previous studies showing strong expression of this feedback regulator gene in adult rat 5-HT neurons [21] and as shown here it is expressed strongly in a serotonergic pattern in the adult mouse dorsal raphe (Figure 3C). In contrast, its expression is undetectable in other brain monoaminergic neuron types. This suggests that BH4 production in 5-HT neurons is especially sensitive to Gfrp-dependent BH4 negative feedback and Gfrp-dependent L-Phe stimulation of BH4 biosynthesis [21]. One possibility is that this provides for rapid and precise adjustments in 5-HT synthetic rates in response to changing behavioral and metabolic states. The *Pcbd1* paralog, *Pcbd2*, has a relatively low expression level (*Pcbd1* average probe intensity: 1897±239, *Pcbd2*: 384±13) suggesting *Pcbd1* plays the major role in BH4 regeneration in 5-HT neurons. Although, *Gchfr*, *Qdpr*, and *Pcbd1* were robustly expressed at E12.5, *Gch1*, *Pts* and *Spr* expression was relatively low (Figure 3B). Yet, the level of *Gch1*, *Pts*, and *Spr* expression must be adequate to support evident abundant synthesis of 5-HT at this fetal stage. ISH confirmed that by adulthood, *Gch1* is indeed strongly expressed in the mouse dorsal raphe (Figure 3D).

Besides *SR*, several other enzymes can catalyze the last three steps of the *de novo* BH4 synthesis pathway. Of these, enzymes encoded by *Akr1b3*, and *Cbr1* but not *Akr1c18* are robustly expressed at E12.5 suggesting multiple BH4 synthesis pathways may be involved in 5-HT synthesis in immature 5-HT neurons (Figure 3B, Figure 1)). Finally, the regeneration/salvage gene, DHFR, is weakly expressed in immature 5-HT neurons (Figure 3B).

Interestingly, our array experiments revealed a severe loss of *Slc22a3* expression in *Pet-1*$^{-/-}$ 5-HT neurons thus demonstrating that Pet-1 controls not simply high affinity, low capacity 5-HT transport (Sert) but also low affinity, high capacity transport (Oct3) (Figure 3B). Our findings further revealed substantially reduced expression of *Gchfr*, and *Gch1* (Figure 3B). Therefore, Pet-1 is a major transcriptional regulator of the rate limiting enzymatic and post-translational regulatory steps for BH4 synthesis. Given the substantial loss of expression of *Gchfr* and *Gch1* expression in *Pet-1*$^{-/-}$ 5-HT neurons, it is somewhat surprising that the expression levels of the other BH4 synthetic genes, *Spr* and *Pts* were unchanged. Perhaps the very low expression of *Pts* and *Spr* near background levels detected for these genes at E12.5 precluded detection of Pet-1 dependency as was likely the case for *Htr1b*. Further studies of Pet-1 dependency for these two genes at later stages of life will be required. The array results also indicated a role for Pet-1 in regulation of the BH4 regeneration pathway as *Qdpr* levels were significantly reduced in *Pet-1*$^{-/-}$ 5HT neurons (Figure 3B). However, loss of Pet-1 had no effect on *Pcbd1, Pcbd2, Cbr1, Akr1b3, Akr1c18,* and *Dhfr* expression level. Given the strong and comparable levels of expression of *Pcbd1, Cbr1, and Akr1b3* in the wild type and mutant arrays we conclude that Pet-1 is not necessary for their expression at least at E12.5. One possibility is that Lmx1b or Gata-3 is required for expression of these other BH4 synthesis, regeneration, and salvage pathway genes. Alternatively, all three transcription factors may play compensatory roles in the expression of these genes so that removal of a single regulatory partner has little or no effect on expression of these genes. A final possibility is that the more broadly expressed broadly functional BH4 genes such as *Akr1b3* are controlled by another gene regulatory network.

Together these findings reveal an extended battery of serotonergic genes under the control of Pet-1. *Slc22a3* and the BH4 pathway genes are expressed in many cell types in brain and periphery while Pet-1 expression in the brain is restricted to the 5-HT lineage. Therefore, it seems likely that the cis regulatory regions of the *Slc22a3* and BH4 genes possess a Pet-1 binding module that functions specifically to direct their expression to 5-HT neurons. Either Lmx1b and/or Gata-3 or other unknown factors may also control expression of *Gchfr, Gch1, Qdpr* and may account for the residual expression of these BH4 pathway genes in *Pet-1*$^{-/-}$ 5-HT neurons.

Although we obtained highly concordant array results among our biological replicates for *Slc22a3, Gch1, Gchfr* and *Qdpr* dependence on Pet-1, we selected *Slc22a3* for RT-qPCR verification using dissected E12.5 neural tubes (Figure 4A). Consistent with the array results, we detected significantly reduced expression of *Tph2*, *Slc6a4*, and *Slc22a3* but not *Lmx1b* in *Pet-1*$^{-/-}$ neural tubes (Figure 4A).

We next investigated adult *Slc22a3* expression with *in situ* hybridization (ISH). Consistent with the Allen Brain Atlas ISH analysis of *Slc22a3*, we found a highly restricted serotonergic raphe pattern of expression for this gene in the midbrain and pons (Figure 4B, left panel and data not shown) suggesting a highly selective serotonergic role for this gene in these brain regions.

Analysis of *Slc22a3* expression in in adult *Pet-1*$^{-/-}$ mice revealed a striking nearly complete loss of its expression (Figure 4B, right panel). This finding was surprising given that

expression of other serotonergic identity features such as Tph2 and 5-HT itself are not absolutely dependent on Pet-1 for their full expression in about 20–30% of 5-HT neurons [7, 13], which is shown here in Figure 4C for comparison to the *Slc22a3* dependency on Pet-1. Thus, our *Slc22a3* findings suggest that whether or not some 5-HT neurons are resistant to loss of Pet-1 depends on specific identity features expressed in these neurons. We note, however, that the residual expression of *Tph2* in individual *Pet-1*$^{-/-}$ 5-HT neurons (Figure 4C, lower panels) appears to be substantially weaker than its expression level in individual wild type 5-HT neurons suggesting Pet-1 controls *Tph2* expression in all 5-HT neurons.

To further investigate *Slc22a3* dependency on Pet-1, we systematically investigated its expression throughout the entire dorsal raphe nucleus of *Pet-1*$^{-/-}$ adults. ISH was performed on every other 25μm section spanning the entire dorsal raphe. As shown in Figure 5A–H, we found a nearly complete elimination of *Slc22a3* expression at all levels of the dorsal raphe in *Pet-1*$^{-/-}$ mice. Residual expression, if any, was confined to scattered cells in numbers far lower than the number of mutant neurons still expressing *Tph2* or 5-HT in the *Pet-1*$^{-/-}$ brain (Figure 4C). These findings indicate that in the case of the *Slc22a3* identity feature its expression is completely dependent on Pet-1 as virtually no 5-HT neurons are resistant to loss of Pet-1. Thus, few if any 5-HT neurons acquire their complete adult identity in the absence of Pet-1 and the number of Pet-1 resistant 5-HT neurons that exist is far fewer than previously thought [7, 13].

To extend this analysis to an additional key identity gene we investigated *Htr1a*'s dependency on Pet-1. ISH throughout the dorsal raphe indicated a similar severe loss of *Htr1a* expression in *Pet-1*$^{-/-}$ mice (Figure 5I–P). Few if any cells in the mutant dorsal raphe expressed *Htr1a* at levels comparable to levels of *Htr1a* in wild type dorsal raphe or levels of residual *Tph2* expression in cells of the *Pet-1*$^{-/-}$ dorsal raphe (Figure 4C). Instead, a low uniform level of blue precipitate was present in cells of the mutant dorsal raphe, which might be background ISH signal, low residual expression in mutant 5-HT neurons or normal expression in non-serotonergic cells. Thus, in the case of the *Htr1a* identity feature far fewer 5-HT neurons are resistant to loss of Pet-1 compared to the number of Pet-1 resistant 5-HT neurons in the case of the Tph2 gene. Further gene expression studies are likely to reveal other serotonergic identity features whose expression is dependent on Pet-1 in all brain 5-HT neurons.

In summary, our findings provide additional insight into the regulatory strategies that enable 5-HT synthesis and reuptake in the brain. We show that Pet-1 globally controls acquisition of the brain's capacity for 5-HT synthesis by coordinating coexpression of *Tph2*, *Ddc*, and genes required for BH4 cofactor synthesis, regeneration, and post-translational negative feedback (Figure 6). Thus, Pet-1 controls all of the known rate-limiting enzymatic (Tph2, Gch1) and post-translational regulatory (Gfrp) steps that determine the level of mammalian brain 5-HT synthesis. In addition, we show that Pet-1 globally controls acquisition of 5-HT reuptake in dorsal raphe 5-HT neurons by coordinating expression of high affinity, low capacity transport via Sert and low affinity, high capacity transport via Oct3 (Figure 6). We show that few, if any 5-HT neurons in the dorsal raphe are resistant to loss of Pet-1 for expression of *Slc22a3*. Similarly, Pet-1 is indispensable for expression of *Htr1a* in what

appears to be all or nearly all 5-HT neurons of the dorsal raphe that normally express this gene. These findings indicate that while some 5-HT neurons are not absolutely dependent on Pet-1 for expression of Tph2 virtually all dorsal raphe 5-HT neurons require Pet-1 for expression of other key identity genes. Therefore, few if any 5-HT neurons in the dorsal raphe are truly resistant to loss of Pet-1 for their neurochemical differentiation.

Methods

Animals

Pet-1$^{-/-}$ mice are described in Hendricks et al. 2003. All *in situ* hybridizations were performed on age and sex matched adult mice 6–8 weeks of age unless otherwise stated. The National Institutes of Health guide was followed for the care and use of laboratory animals. All experiments were approved by the CWRU School of Medicine Institutional Animal Care.

Perfusion and Sectioning

Mice were anesthetized with Avertin (0.5 g tribromoethanol/39.5 ml H2O + 0.31ml tert-amyl alcohol) and transcardially perfused with saline followed by cold 4% paraformaldehyde (PFA). Brains were extracted and post-fixed for 2 hrs and incubated overnight (O/N) in 30% sucrose-PBS solution at 4°C. Floating 25μm coronal brain sections of the dorsal raphe serotonin system were taken, mounted on SuperFrost Plus slides (Fisher Scientific), and dried in a vacuum chamber for at least 1hr before use.

In Situ Hybridization (ISH)

Digoxigenin (Roche Diagnostics, Indianapolis, Indiana) labeled antisense RNA probes (~600bp) were synthesized using cDNA fragments of *Slc22a3*, *Htr1a*, *Gch1*, *Gchfr* or *Tph2* that were PCR amplified with reverse primers containing bacteriophage T3 promoter sequences at their 5′ ends. A previously published *in situ* hybridization protocol was followed [(10)].

Quantitative Real-time PCR (RT-qPCR)

RNA was isolated from E12.5 *Pet-1*$^{+/-}$ and *Pet-1*$^{-/-}$ animals from the rostral serotonin system using PureLinkTM RNA Mini Kit (Ambion by Life Technologies, Carlsbad, California). Purified RNA was converted to cDNA by PCR using the Transcripter FirstStrand cDNA Synthesis Kit (Roche Diagnostics, Indianapolis, Indiana) and stored at −20°C. qPCR was performed in triplicate using Fast StartTM Universal Syber Green ROX Master solution (Roche Diagnostics, Indianapolis, Indiana). Samples were normalized to β-actin.

Immunohistochemistry (IHC)

Fluorescent immunohistochemistry was performed using a polyclonal primary rabbit antibody against 5-HT (1:1000, ImmunoStar, Hudson, WI) or a primary chicken antibody against GFP (1:1000, Abcam, San Francisco, Ca) O/N at 4°C and an anti-rabbit or anti-chicken Alexa Fluor®488 secondary antibody (1:500, Invitrogen by Life Technologies,

Carlsbad, California) for 1hr at RT in the dark. A standard IHC protocol was used [6]. Fluorescent images were taken using SPOT RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) using an Olympus Optical BX51 microscope (Center Valley, PA).

FACS of YFP$^+$ cells

The rostral hindbrain domain from the mesencephalic flexure to the pontine flexure of either *ePet-EYFP*$^{+/+}$ and *ePet-EYFP*$^{Pet\text{-}1-/-}$ embryos was dissected and then treated with 0.25% trypsin-EDTA (Life Technologies) to dissociate cells as described previously [33]. Cells were filtered through a 40μm filter and sorted using a Becton Dickinson FACS Aria digital cell sorter with an argon laser (200mW @ 488nm). Cells were sorted directly into Trizol (Invitrogen) for RNA extraction. Approximately 7000 YFP+ cells were isolated from +/+ or *Pet-1*$^{-/-}$ embryos. Each of the 4 biological replicates (4 +/+,4 *Pet-1*$^{-/-}$) consisted of between 20–30 thousand cells from independent litters.

Microarray

Total RNA was isolated after the addition of 20μg of glycogen (Invitrogen) using phenol chloroform extraction. RNA amplification and cDNA libraries were prepared using the Ambion®WT Expression Kit (Life Technologies) according to the manufacturer's protocol. 5.5μg of single-stranded DNA was fragmented and labeled using Affymetrix GeneChip® WT Terminal Labeling Kit. Probes were hybridized overnight at 45°C to a GeneChip Mouse Gene 1.0 ST Array (Affymetrix). After hybridization, chips were washed in a Genechip Fluidics Station (Affymetrix) and scanned at high resolution using an Affymetrix High Density GeneChip Scanner 3000. The.CEL files from the 8 chips were normalized using the Robust MultiChip Averaging (RMA) using Affymetrix® Expression ConsoleTM Software version 1.1.

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Abbreviations

5-HT	serotonin
BH4	6R-L-erythro-5,6,7,8-tetrahydrobiopterin
Tph2	Tryptophan hydroxylase 2
Sert	serotonin transporter
Oct3	Organic cation transporter 3
Gch1	GTP cyclohydrolase I
Gchfr	GTP cyclohydrolase feedback regulator

Qdpr	quinoid dihydropteridine reductase
ISH	in situ hybridization

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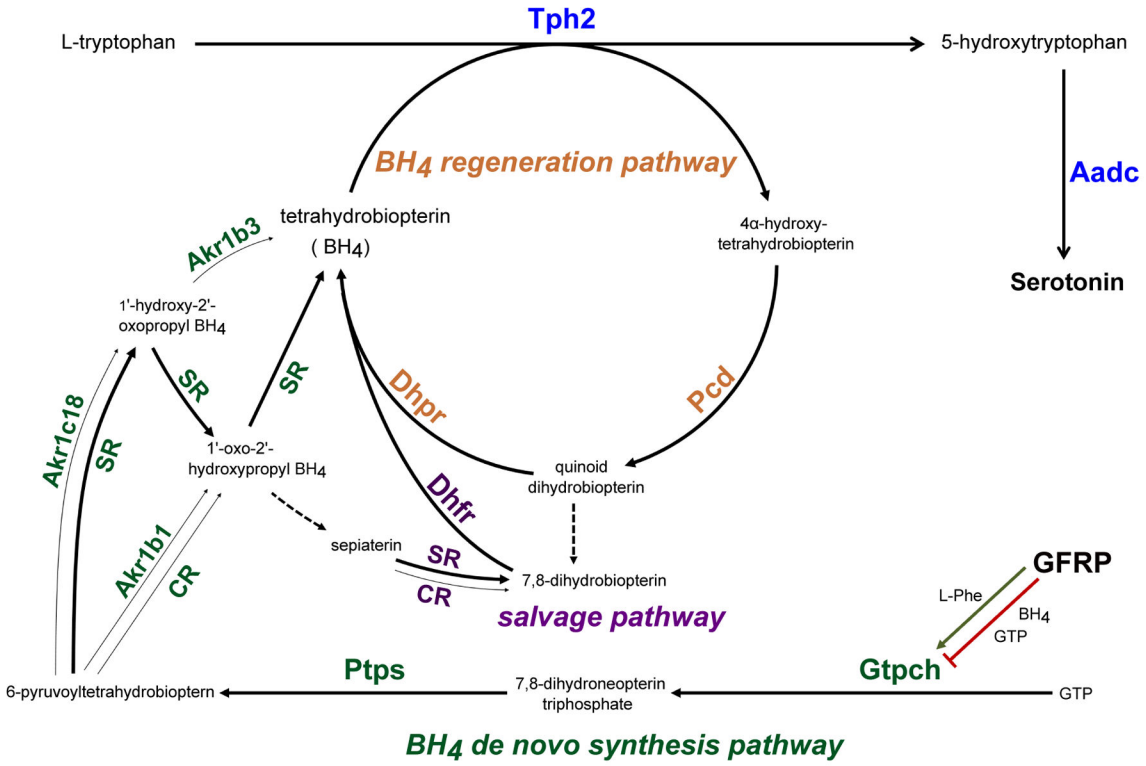

Figure 1.

Schematic of BH4 *de novo* synthesis, salvage, and regeneration pathways and its role in 5-HT synthesis. Tph2, Tryptophan hydroxylase 2; Aadc (*Ddc*), aromatic L-amino acid decarboxylase; Gfrp (*Gchfr*), GTP cyclohydrolase I feedback regulator; Gtpch (*Gch1*), GTP cyclohydrolase; Ptps (*Pts*), 6-pyruvoyl-tetrahydropterin synthase; SR (*Spr*), sepiapterin reductase; Akr1c3, aldo-keto-reductase family 1 member 3; Akr1b1, aldo-keto-reductase family 1 B1; CR (*Cbr1*), carbonyl reductase; Dhfr dihydrofolate reductase; Dhpr (*Qdpr*), dihydropteridine reductase; Pcd (*Pcbd1*, *Pcbd2*), pterin-4 alpha-carbinolamine dehydratase. Dashed lines indicate non-enzymatic steps.

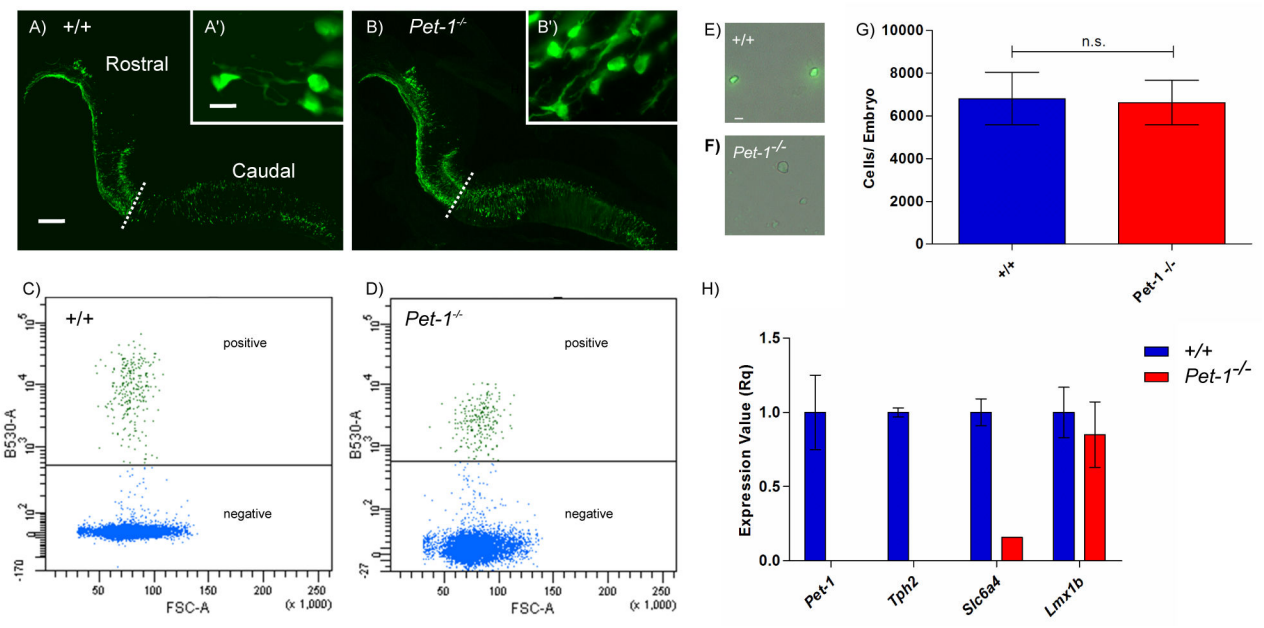

Figure 2.

Isolation of *ePet-EYFP*$^{+/+}$ and *ePet-EYFP*$^{Pet\text{-}1-/-}$ 5-HT neurons. (A–B) Sagittal sections of E12.5 embryonic hindbrain. Dashed line indicates division between rostral and caudal 5-HT neurons. (C–D) Flow cytometry data of sorted YFP^{+} cells. X-axis Forward Scatter Area (FSC-A). Y-axis 488nm fluorescent intensity. (E–F) Sorted 5-HT neurons. (G) Number of YFP+ cells per embryo collected from either +/+ or *Pet-1*$^{-/-}$ animals (p=1.00). (H) RT-qPCR validation of selected 5-HT genes in +/+ vs *Pet-1*$^{-/-}$ samples. Scale, 200μm; zoom, 10μm.

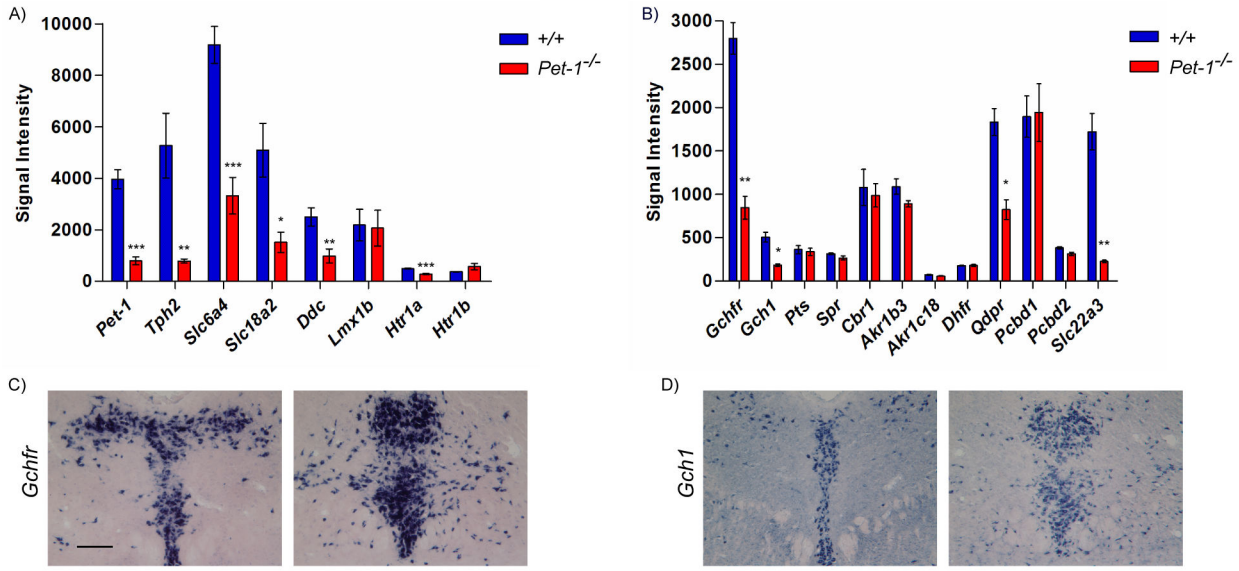

Figure 3.

Microarray analyses. (A) Signal intensities of 5-HT neuron-type identity genes. (B) Signal intensities of BH4 pathway genes and *Slc22a3*. (C) *In situ* hybridization of *Gchfr* and (D) *Gch1* probes at two rostrocaudal levels of the dorsal raphe of 3 week old mice. Scale, 200μm. Genes were analyze by a Student's t-test followed by a Bonferroni correction. Corrected p-value: *p<0.05, **p<0.01, ***p<0.001.

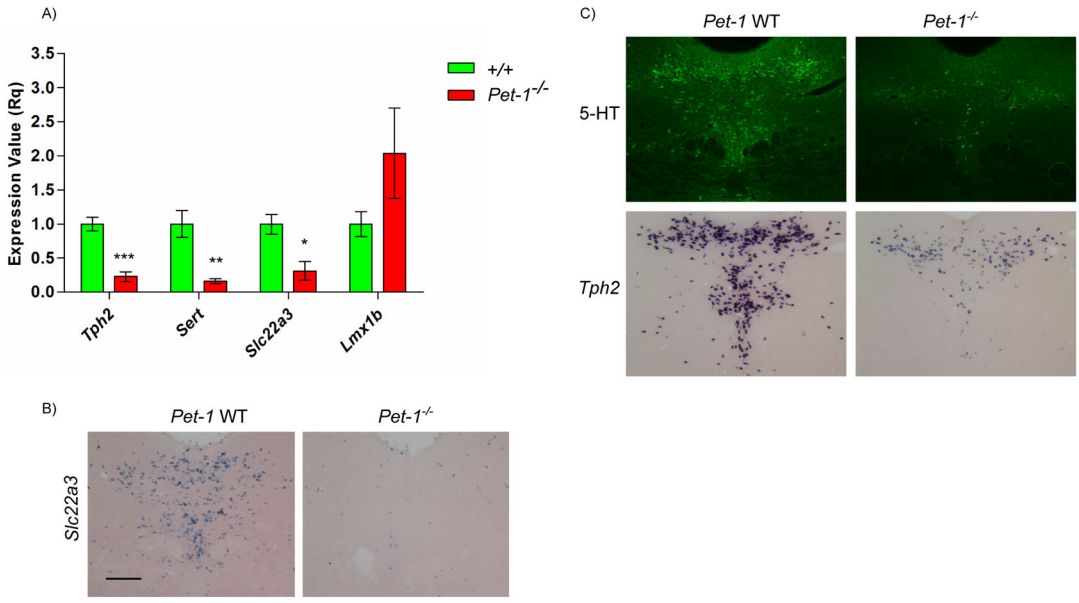

Figure 4.

Slc22a3 expression. (A) RT-qPCR analyses of 5-HT genes with RNA obtained from unsorted hindbrain dissections of E12.5 *Pet-1*$^{+/-}$ or *Pet-1*$^{-/-}$ embryos. (B) Comparative *in situ* hybridization of *Slc22a3 probe* in +/+ and *Pet-1*$^{-/-}$ mice. (C) 5-HT immunohistochemistry and *Tph2 in situ* hybridization in +/+ and *Pet-1*$^{-/-}$ adult mice. Scale, 200μm. Genes were analyze by a Student's t-test. p-value: *p<0.05, **p<0.01, ***p<0.001.

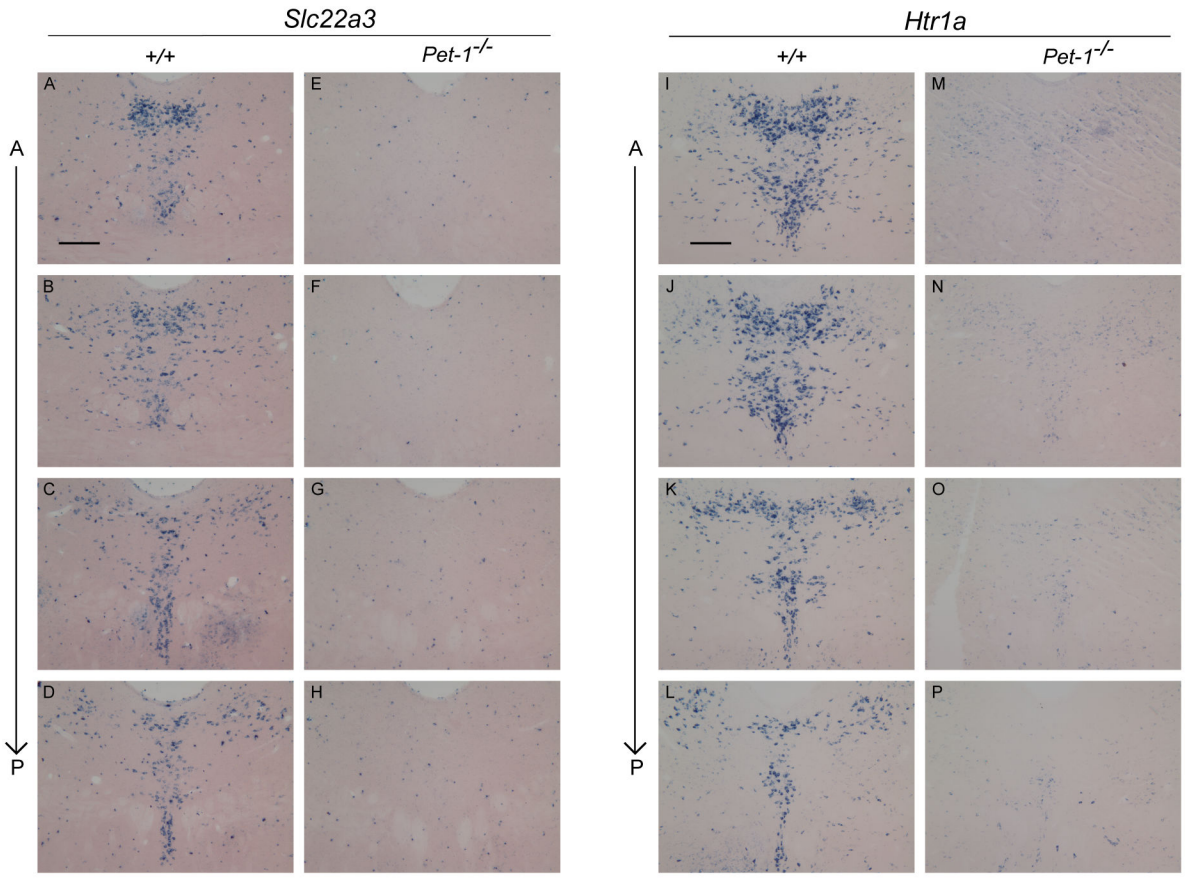

Figure 5.

Slc22a3 and *Htr1a expression in the adult dorsal raphe.* 25μm coronal brain sections processed by *in situ* hybridization and developed for 15hrs using an (A–H) *Slc22a3* or (I–P) *Htr1a* probe on either wild type or *Pet-1*$^{-/-}$ tissue sections. Alternate sections through the entire dorsal raphe were used for each probe. (A) anterior; (P) posterior. Scale, 200μm.

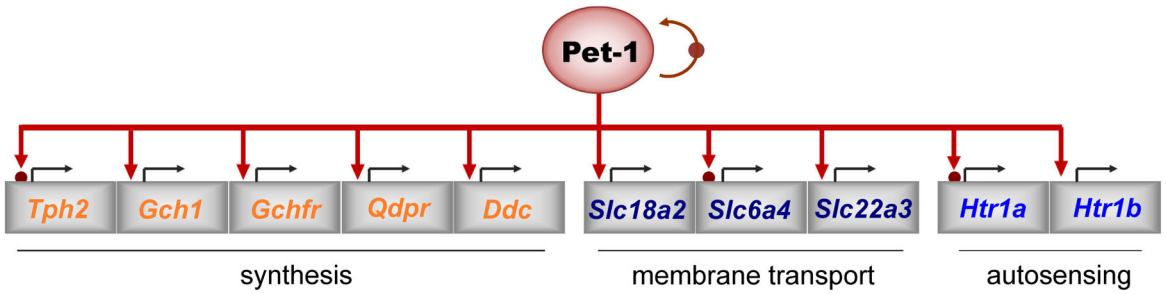

Figure 6.

Pet-1 control of the 5-HT neuron-type gene battery. Pet-1 coordinates expression of genes required for 5-HT synthesis (orange), vesicular transport and reuptake (purple) and autoreceptor function (blue). Filled red circles indicate direct control of genes as determined by chromatin immunoprecipitation, reporter assays and in vitro mobility shift assays. Other genes in the schematic may also be direct targets of Pet-1 but have not yet been investigated.